CERTIFICATE OF FACSIMILE TRANSMISSION

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Ana Lavsky
Name

December 24, 2003
Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors: Choy-Pik Chiu & Robert Kay	Art Unit: 1636
Filing Date: November 21, 2001	Examiner: Quang Nguyen, Ph.D.
Serial No: 09/990,522	
Docket: 097/002	
Title: TOLERIZING ALLOGRAFTS OF PLURIPOTENT STEM CELLS	

DECLARATION UNDER 37 CFR § 1.132
BY ANISH S. MAJUMDAR, Ph.D.

Commissioner for Patents
Alexandria VA 22313

Dear Sir:

I, ANISH MAJUMDAR, do hereby declare as follows:

1. I am Associate Director of Immunology at Geron Corporation. [PREVIOUS EXPERIENCE].

A copy of my *curriculum vitae* accompanies this Declaration.

2. While at Geron, I have led projects to build an oncolytic virus based on the telomerase promoter (Gene Ther. 8(7):568-78, 2001), and to develop a telomrease anti-cancer vaccine (Cancer Gene Ther. 10(3):239-49, 2003).

More recently, I have been investigating the immunological aspects of human embryonic stem cells, and tissue cells derived from them. We are designing strategies to improve acceptance of xenografts and allografts of neural and cardiac tissue made from hES cells.

3. This patent application describes a system we have been working on in which hES cells are differentiated into two cell populations: the first, a toleragenic cell population (exemplified by hematopoietic or mesenchymal cells); the second, a therapeutic cell population (exemplified by neurons, cardiomyocytes, islet cells, and other alternatives). The idea is that the first cell population will render the host tolerant to the histocompatibility type of the hES cell line being used. The second cell population can then be given for regenerative medicine, with reduced risk of allograft rejection.

4. I understand the Examiner has questioned whether hES derived cells can be used to induce tissue tolerance in this fashion.

Attached to this Declaration are three pages of data that provide results of experiments that are instructive in this regards.

5. The ability of hES cells to induce proliferation of allogeneic T cells was measured in a mixed lymphocyte reaction (MLR). It was found that hES cell lines are unable to induce allo-reactivity in primary human T cells, even after stimulation with interferon gamma.

Peripheral blood leukocytes were isolated from heparinized blood using a density gradient. To enrich for T lymphocytes, separated cells were incubated for 2 h at 37°C, and the non-adherent cells were collected and frozen for later use. Dendritic cells (DCs) were prepared by culturing the remaining adherent cells for 7 d in medium containing human recombinant GM-CSF and IL-4.

The mixed lymphocyte reaction was performed as follows: stimulator cells were irradiated, and then 1×10^5 to 1×10^2 cells were plated in 96-well round bottom plates. Responder PBMC or T cells were added at a concentration of 1×10^5 per well, and the plates were cultured for 5 days. The wells were then pulsed with [^{3}H]thymidine, harvested, and counted.

Figure 1 shows the results of the direct MLR where undifferentiated hES cells were used as stimulators at a 1:10 ratio to responding T cell enriched (monocyte depleted) peripheral blood

leukocytes (mean stimulation index ± SEM of multiple wells from 3 donors). The positive control are dendritic cells (DC) obtained from an allogeneic donor, which stimulated a strong proliferative response by the T lymphocytes.

However, none of the three hES cell lines tested were able to stimulate T cell proliferation. In contrast to what would be predicted for mature cells like cardiomyocytes, the early stage hES cells do not provoke immune reactivity in allogeneic responder cells.

6. We then did an experiment that shows hES cells are not just inert and inactive participants — they actively suppress immunoreactivity, even between stimulators and responders that are otherwise highly immunogenic.

Mixed lymphocyte reactions were set up that contained three components: human irradiated PBL stimulator cells, allogeneic human dendritic cell (DC) responders prepared as in the previous example from a different donor, and irradiated modulator cells, being tested for their ability to inhibit the reaction between the PBL stimulators and the DCs. Responder T cells (1×10^5) were cultured in combination with 1×10^5 irradiated human fibroblasts or hES cells. Subsequently, 1×10^4 irradiated dendritic cells were added per well. After 5 days culture, the cells were pulsed with [^{3}H]thymidine, washed, and counted.

Figure 2 shows the results (mean ± SEM). The PBLs used in this experiment invoked a strong proliferative response to the DCs, as indicated by uptake of tritiated thymidine. Adding BJ fibroblasts into the reaction had no significant effect. However, undifferentiated hES cells of the H1, H7 and H9 lines all showed an ability to quench the reaction to about 1/3 to 1/4 of the control value.

Figure 3 shows a subsequent experiment in which the inhibitory effect of the hES cells was titrated. Serial reduction in the number of hES cells resulted in a gradual loss of the inhibitory effect, showing that inhibition by hES cells of alloactivation in a mixed lymphocyte reaction is dose-dependent (Panel B). The MLR was inhibited at a hES cell:T cell ratio of 1:1 or 1:3.

This demonstrates that hES cells actually prevent immune cells from reacting against stimulators that otherwise have very strong immunostimulatory capacity.

7. A subsequent experiment illustrates that progenitor cells made from hES cells retain the ability of these cells to prevent stimulation of autologous T lymphocytes.

Early stage progenitor cells were obtained by established methods. hES cells were cultured in suspension for 4 days so as to form embryoid bodies. They were plated onto

poly-L-lysine/fibronectin-coated plates in a medium containing N2 supplement, B27 supplement, EGF, bFGF, PDGF-AA, and IGF-1, and cultured for 3 days so as to make neural progenitors (WO 03/000868). The cells were harvested by brief trypsinization, plated onto poly-L-lysine, and then cultured in medium containing B27 supplement, NT-3, and BDNF for 5 days. After replating, they were cultured with, BDNF, EGF, and bFGF. They were fed three times per week, and replated every 7 days.

Figure 4 shows MHC Class I expression in the differentiated progenitor cells, evaluated by FACS analysis (Upper panels). Compared with undifferentiated hES cells, the neural progenitor population derived from both hES lines express higher levels of MHC Class I antigens on their surface.

However, this apparent increase in MHC Class I antigen expression in the progenitors did not increase their ability to stimulate allogeneic T cell proliferation (Lower panel). In fact, the hES derived progenitors failed to induce significant T cell proliferation by responder populations from two donors. Undifferentiated hES cells were similarly poor stimulators. In contrast, normal BJ fibroblasts induced moderate levels of T cell proliferation, compared with the dendritic cell positive control.

These results show that some of the important immunologic properties of hES cells persist in progenitor cells during the early stages of differentiation.

8. I hereby declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

12-23-03
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